IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Neuberger, T. et al. EXAMINER: Kwon, Brian Yong S.

SERIAL NO.: 09/827,666 ART UNIT: 1614

Filed: April 6, 2001

For: COMPOSITIONS AND METHODS FOR PROMOTING TISSUE REGENERATION

DECLARATION UNDER 37 C.F.R. 1.132

COMMISSIONER FOR PATENTS
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ALEXANDRIA, VIRGINIA 22313-1450

SIR:

I, TIMOTHY NEUBERGER, hereby declare and state that:

1. I am a Group Leader in the Department of Discovery Operations at Vertex Pharmaceuticals, Inc., having received my Ph.D. degree from the University of Vermont in 1989. After that I was a postdoctoral fellow at the Medical College of Virginia in the Department of Biochemistry.

2. My full curriculum vitae is attached hereto as Exhibit A.

3. My principal area of research is neuroscience and more recently my current research is in cystic fibrosis, and among other positions I serve as reviewer for several funding agencies, including the National Institutes of Health in the Brain Disorders and Clinical Neurosciences Study Section, The National Science Foundation and the Veterans Affairs Merit Review Board.

4. In the course of my activities, I have been listed as inventor on several patent applications, including the one noted above entitled "COMPOSITIONS AND

METHODS FOR PROMOTING TISSUE REGENERATION", having U.S. Serial Number 09/827,666, filed on April 6, 2001.

5. I have reviewed the disclosure of the present application, with particular emphasis on the enablement of the invention as claimed and more particularly with respect to the use of the compounds of the invention for growing neural cells and promoting their differentiation in vitro and in vivo. Given the methods provided for obtaining the cells for such studies, using both neural and non-neural tissue, it would be possible for one skilled in the art to use this information to test compounds or small molecules such as those described in the present application for effects on neuronal cell growth and differentiation. In my opinion, the disclosures of this current application are sufficient to enable one skilled in the art to make or use the invention as described therein and concomitantly provide to the practitioner teachings that could be applied for the indicated purposes.

6. In this regard, and in corroboration of the disclosure of this current application, I have conducted experiments which confirm the disclosure in this application and which support the utility of the compounds described in this application for identification of the small molecules described in this application that promote growth and differentiation of neuronal cells and which may be used to treat neurons injured due to disease, surgery, or other factors such as excitotoxic agents. Experiments were conducted which demonstrate such effects and these studies are summarized here.

7. CNS (brain) tissue was harvested from embryonic day 18 (E-18) rat pups and the tissue was dissociated to release isolated neural cells. In one study, the excitotoxic agent glutamate was added to all the wells except control wells, which received media minus glutamate. In one study, which corresponds to Example 1 in the present application, the cells derived from the E-18 pups were treated on in vitro day 19 (after being treated with glutamate) with either N-[4-[(4-fluorophenyl)sulfonyl]phenyl]acetamide dissolved in 2-hydroxypropyl β cyclodextrin or treated with the vehicle control (2-hydroxypropyl β cyclodextrin) alone. The final

concentrations of the compound were 100 μg/ml, 10 μg/ml, 1 μg/ml and 0.1 μg/ml or 10 μg/ml, 1 μg/ml 0.1 μg/ml and 0.01 μg/ml. Cultures were maintained for up to an additional 10 weeks. One to two 24 well cluster plates were immunostained every week using known antibody markers including anti-eNCAM, anti-β-tubulin, anti-MAP II, anti-neurofilament and anti-low affinity NGF receptor. These antibodies were selected because they are accepted markers for detecting neurons in different stages of development and differentiation (See, e.g., A Caceres, L I Binder, M R Payne, P Bender, L Rebhun, and O Steward, "Differential subcellular localization of tubulin and the microtubule- associated protein MAP2 in brain tissue as revealed by immunocytochemistry with monoclonal hybridoma antibodies,"J. Neurosci. 1984 4: 394-410). The results demonstrate a profound increase in cells expressing all of the above noted markers following incubation with the drug. However, for illustrative purposes, only the eNCAM and beta tubulin photographs are enclosed here. The optimal dose was approximately 0.1 μg/ml to 1 μg/ml N-[4-[(4-fluorophenyl)sulfonyl]phenyl]acetamide and the results of this study are attached here as photographs of the cell cultures. The drug N-[4-[(4-fluorophenyl)sulfonyl]phenyl]acetamide is referred to in these photographs as Acord-00001.

8. Another study was conducted using post natal day 5 rat pups, which is equivalent to the study reported in the present application in Example 2. Unlike the first study described above, the neural cell cultures were not treated with glutamate. Following isolation of cortical astrocytes from the brains of the day 5 rat pups, the cells were incubated in the presence of N-[4-[(4-fluorophenyl)sulfonyl]phenyl]acetamide or with the vehicle control. Using immunostaining protocols described in Example 2 of the application, 24 well cluster plates were immunostained once per week for up to 10 weeks after treatment with N-[4-[(4-fluorophenyl)sulfonyl]phenyl]acetamide using antibodies against eNCAM, β-tubulin, MAP II, phosphorylated neurofilament or low affinity NGF receptor. As in the first experiment, dramatic differences were observed in the cultures treated with N-[4-[(4-fluorophenyl)sulfonyl]phenyl]acetamide as compared to the vehicle controls with respect to staining with anti-eNCAM, anti-β tubulin, anti-MAP II, anti-

neurofilament and anti-low affinity NGF receptor. For illustrative purposes, the cultures stained with anti-β tubulin, anti-eNCAM and anti-MAP II are shown here.

9. Another study was done to assess the effects of N-[4-[(4-fluorophenyl)sulfonyl]phenyl]acetamide on the ability of aged astrocytes to permit the growth of neurons. In this study, astrocytes from postnatal day 2 Sprague Dawley rat cortices were aseptically dissected and astrocytes isolated using the procedure described in Example 4 of the present application. Cells were plated into poly-D-lysine T75 flasks. After 23 days, astrocytes were trypsinized and replated at a density of 500,000 cells per well into poly-D-lysine-coated 24 well plates. Cultures were grown for an additional 20 days in order to let astrocytes mature. This maturation is known to make the astrocytes less permissive for neurite outgrowth (see G. M. Smith, et al., Developmental Biology, 138, 377-390 (1990), and G. M. Smith and J. Silver, Progress in Brain Research, Vol. 78, Chapt. 46, D. M. Cash and J. R. Sladek, ed., Elsevier, 1988). Cortical neurons were isolated by taking mixed cortical cells which were dissociated from embryonic day 18 Sprague Dawley rat embryos. In order to obtain more pure neuronal cultures, cells were subjected to a differential adhesion by pre-plating them on a non-coated bacterial dish (100 mm diameter) for 1 hour. Non-neuronal cells stick to this plate while neurons stay in the supernatant. Neurons were added to the aged astrocytes at a density of 10,000 cells/well in DEME/10% fetal bovine serum. One day later, the cultures were treated with 1 μg/ml and 10 μg/ml of N-4[4-[(4-fluorophenyl)sulfonyl]phenyl]acetamide. 1 week after treatment, cultures were fixed and immunostained with monoclonal anti-tubulin antibody followed by rhodamine-conjugated goat-anti-mouse secondary. Tubulin labels axons and dentrites. Tubulin-positive cell bodies with a process at least two cell diameters long were counted in 5 visual fields under 10X magnification with a Nikon Eclipse 2000 inverted microscope. Results are shown in the attached figures. The aged astrocytes following treatment with N-4[4-[(4-fluorophenyl)sulfonyl]phenyl]acetamide were more able to promote growth of the neurons compared to the non-treated cell cultures.

10. A study was also done to determine whether the compound N-4[4-[(4-fluorophenyl)sulfonyl]phenyl]acetamide could have an effect on neuronal stem cells in

vivo. A study was conducted as described in Example 3 of the present application whereby one group of animals was treated with N-4[4-[(4-fluorophenyl)sulfonyl]phenyl]acetamide and the second group with the vehicle control. Neuronal stem cells are generally isolated from the subventricular zone of the brain. However, while no one has previously demonstrated neuronal stem cells in bone marrow, we isolated a mixed population of cells collected from the bone marrow of drug treated and control animals and used these to inject into the lesion of animals whose spinal cord was contused using standard procedures for this model of spinal cord injury. To do this, bone marrow cells were obtained from both groups of animals 5 days after drug or vehicle treatment and used to treat animals whose spinal cord was contused using the procedure described in Example 3 of the present application. Four weeks after injection of the bone marrow cells into the lesion site, spinal cord tissue was harvested, embedded in paraffin and stained for the Nestin and hematoxylin-eosin/luxol fast blue. Nestin is a known marker of neural precursor cells (Matthew F. McManus, Li-Chun Chen, Inmaculada Vallejo, and Mario Vallejo; "Astroglial Differentiation of Cortical Precursor Cells Triggered by Activation of the cAMP-Dependent Signaling Pathway," J. Neurosci. 1999, 19(20):9004-9015). Control sections lacking the primary antibodies were also processed. As shown in the attached photographs, spinal cord injured animals treated with bone marrow cells derived from drug treated animals resulted in a greater number of cells staining positive for nestin.

11. As shown here, the results are illustrative of profound effects of N-4[4-[(4-fluorophenyl)sulfonyl]phenyl]acetamide on the growth and differentiation of neural cells both in vitro and in vivo as shown by cell surface markers specific for neuronal cells. Using the procedures described in the present application, one of skill in the art could practice the invention as currently claimed. Furthermore, undue experimentation would not be necessary to practice this invention given the guidelines provided in this application. It is my belief that the results shown here and in the application provide proof of enablement of the invention.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18 of the U.S. Code, Section 1001, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Dated: 11/5/04

Timothy Neuberger, Ph.D.

Timothy J. Neuberger, Ph.D.
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San Diego, Ca.
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Education:

University of Vermont, (1989) Ph.D., Cell Biology
Principle Advisor - Carson J. Cornbrooks, Ph.D.
Univ. of New Mexico (1983) B.S., Biology
Parkston High School (1975) Biology

Research & Professional Experience:

02 – present: Group Leader, Discovery Operations, Vertex Pharmaceuticals, San Diego, Ca.
01 – 02: Cell Core Manager, Vertex Pharmaceuticals, San Diego, Ca.
98 – 01: Senior Scientist and leader of Cellular and Molecular Biology and Protein Purification, Acorda Therapeutics Inc. Hawthorne, New York.
94 – 97: Research Scientist, Department of Cell Biology, Amgen Inc. Boulder, Colorado.
94 – 94: Scientist I. Discovery Research, Synergen Inc. Boulder, Colorado.
89 –94: Post-Doctoral Fellow. Dept. of Biochemistry, Medical College of Virginia, Richmond, Virginia.
83 – 89: Graduate School, University of Vermont, Burlington, Vermont.

Honors:

2003 VOCAP (Vertex Outstanding Contributor Award Program) Recipient

Professional Affiliations:

6/93 - pres. American Society for Neurochemistry
1/85 - pres. Society for Neuroscience
8/84 - 5/89 Vermont Chapter for Neuroscience

Professional Services:

Brain Disorders and Clinical Neurosciences (BDCN-E) Study Section, NIH (Study section member)
Brain Disorders and Clinical Neurosciences (BDCN-2) Study Section, NIH (ad hoc reviewer)
National Science Foundation (ad hoc reviewer)
Veterans Affairs Merit Review Board (ad hoc reviewer)

Management, Administrative and Leadership Experience:

- Designed, built and managed large scale cell line production facility at Vertex Pharmaceuticals – San Diego
  - Designed and oversaw construction of cell production facility
  - Identified automation needs for cell production facility, identified vendors and evaluated proposals, wrote factory and site acceptance test specifications. Wrote SOPs and trained personnel to operate automation.
  - Developed innovative solutions to cell culture bottlenecks allowing small staff to produce more than 600 cell based assay plates per day from 20 different cell lines.
  - Established readily scalable methods to dramatically increase staff efficiency and productivity.
  - Increased cell based assay plate production more than 15 fold while stabilizing and enhancing the quality and consistency of the assays. Reduced operating expenses by more than $1.5 million annually.
- Established expertise in large-scale production of primary human and rodent cells.
  - Provide primary human cells from diseased and non-diseased human tissue for primary and secondary assays. Currently, one drug development program is based on the use of primary human cells obtained from the target human tissue.
  - Built sufficient primary cell production capacity to meet the needs of three separate drug development programs.
- Planned, budgeted and established two cell biology laboratories, a protein production and purification facility, a large-scale cell production facility. Designed and oversaw building the first animal facility at Vertex Pharmaceuticals-San Diego. Obtained state permit to house and use animals onsite, established IACUC.
- Recruited and trained junior personnel to operate as a single, highly integrated, efficient team to meet demanding production schedule. Team has a greater than 98% success rate for meeting production demands. In the past 18 months, weekly medicinal chemistry screening cycle was not missed once due to Cell Core.
- Awarded 2 Small Business Innovative Research Grants from the NIH.
- Member of the Brain Disorders and Clinical Neurosciences Study section for 5 years, reviewing more than 100 grant applications. Served on additional study sections as requested. Areas of expertise include development of small molecule, protein and stem cell based therapies for CNS and PNS disorders.
- Two patents issued.

Scientific Experience:

- Developed methods to produce fully functional, differentiated human bronchial epithelial cells isolated from Cystic Fibrosis patients in quantities sufficient to support two medicinal chemistry programs. Using this culture system, I developed secondary assays to predict in vivo efficacy. Development of these methods allowed drug development to be performed using primary cells isolated from the target diseased tissue.
- Developed assays to test compounds identified as potential NURR1 agonists for ability to increase survival and/or expression of tyrosine hydroxylase in neurons isolated from ventral mesencephalon tissue.

- Established protocols and methodologies for production and purification of antibodies and chimeric proteins using mammalian and E.coli expression systems. Developed a new serum free CHO cell culture medium for enhanced protein production. Designed biological activity and stability assays for purified antibodies and chimeric proteins. Using molecular biology techniques, redesigned chimeric proteins and demonstrated enhanced biological activity that resulted in new molecules.
- Expertise in developing novel, cell based assays for screening small molecules, purified antibodies and proteins.
- Cell based assays developed include mixed neural cultures, enriched neuronal cultures, astrocyte cultures, oligodendrocyte cultures and microglial cultures, neural stem cell cultures, bone marrow cultures and endothelial cell cultures. Additional assays were developed using established cell lines. End points included neuron survival and neurite outgrowth, fasciculation and sprouting, oligodendrocyte proliferation, differentiation and survival, astrocyte and microglial activation and cytokine production, stem cell growth and differentiation, neutrophil adhesion, bone marrow cell survival and proliferation, and endothelial cell proliferation. Developed sensitive assays to identify novel complement inhibitors found in primary tissues and tissue culture supernatants. Assays were also used to follow purification of novel complement inhibitors.
- Extensive experience in experimental design, project management, data analysis and presentation of data in both written and oral formats. Teaching experience includes cell biology and histology.

Timothy J. NEUBERGER

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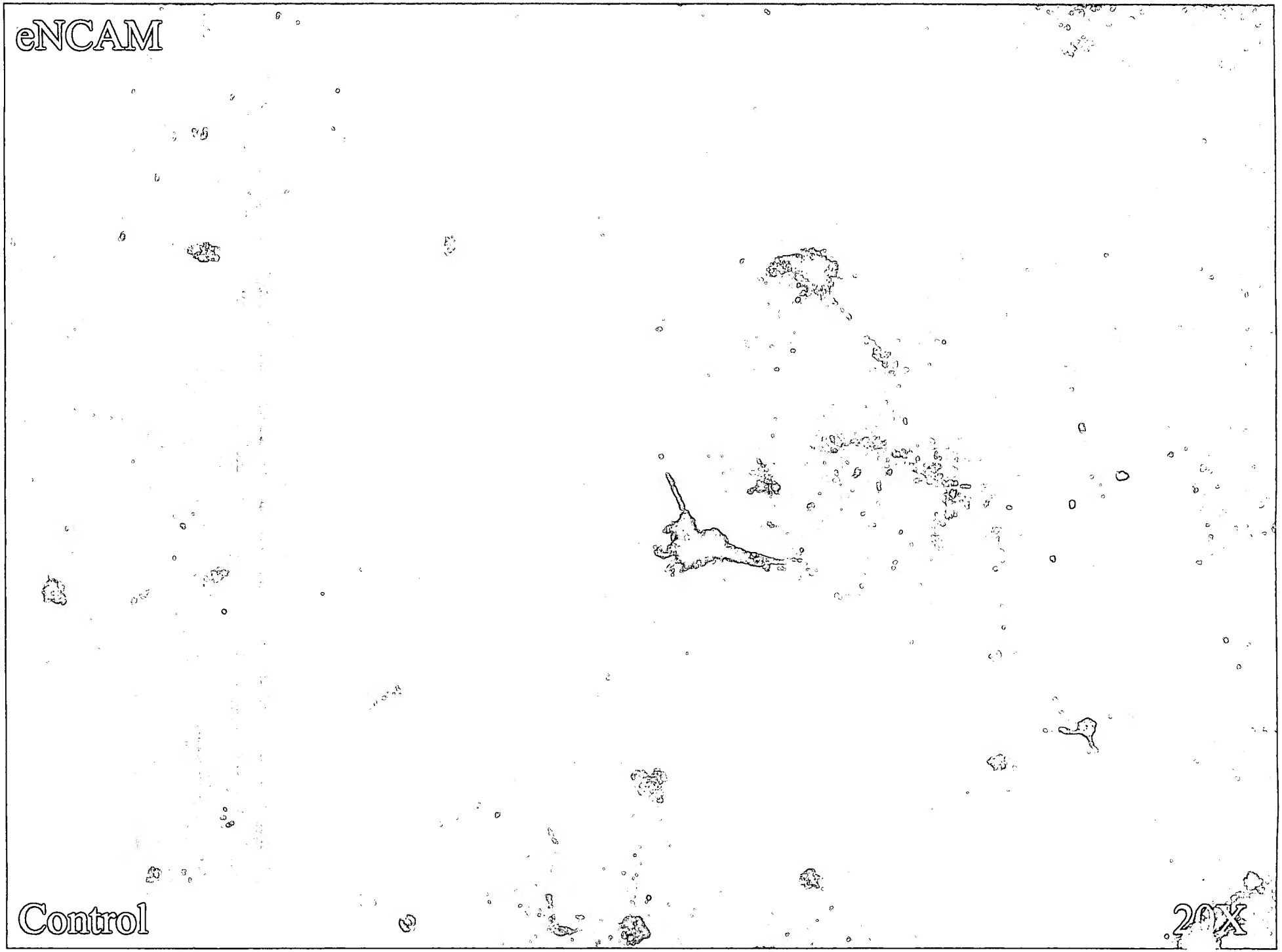
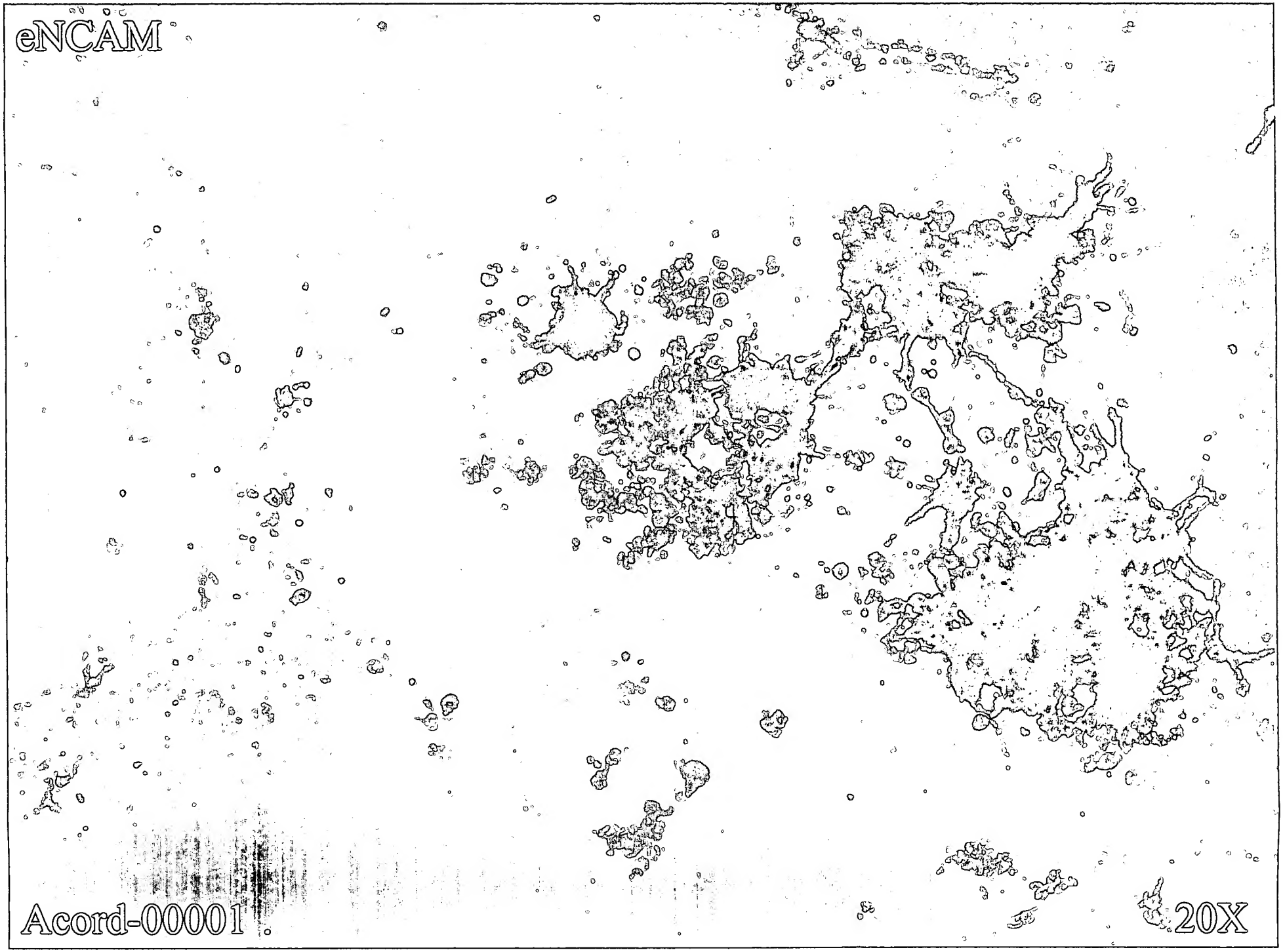
In Vitro Experiments

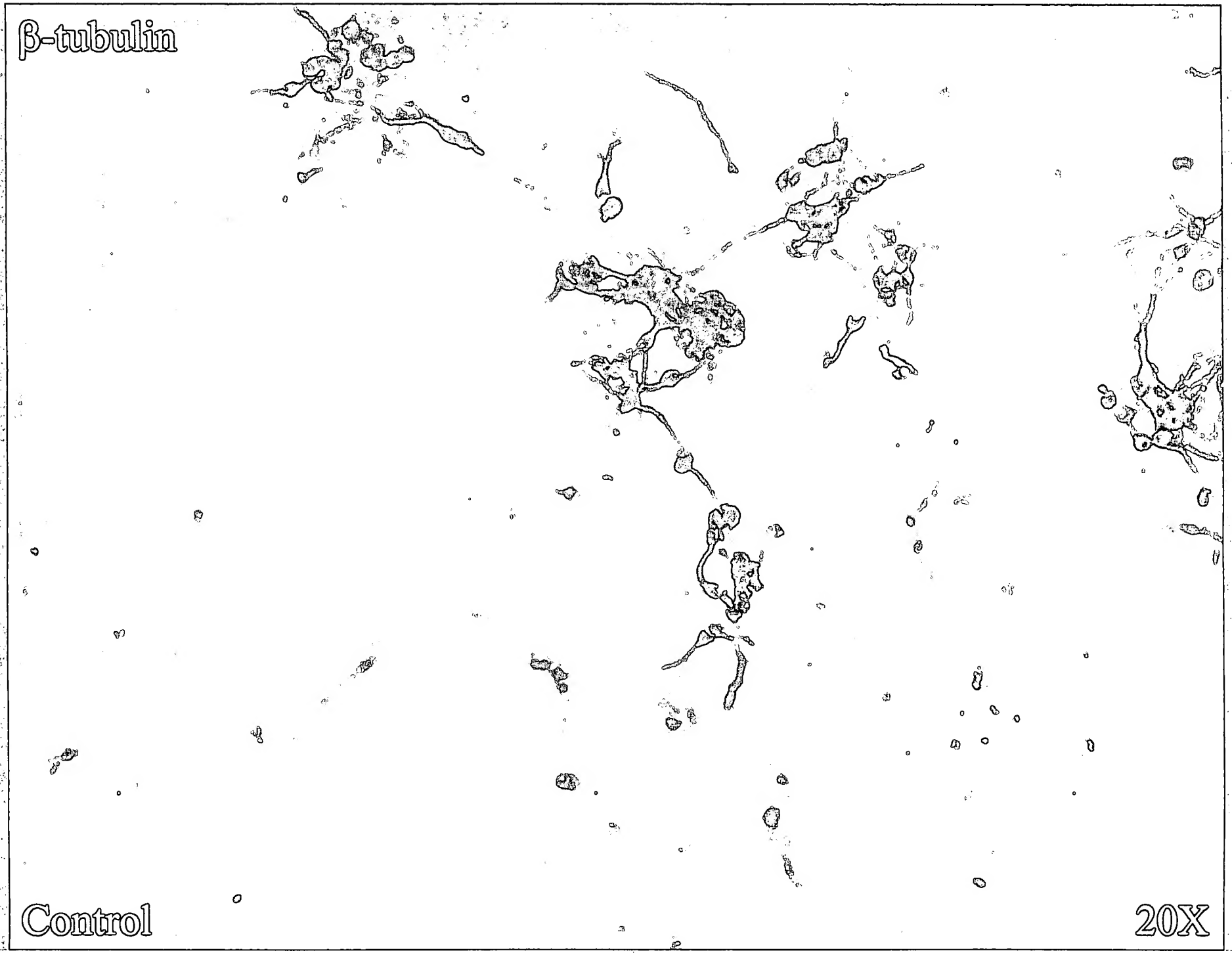
- E-18 cultures one week post treatment
- 1μg/ml Acord-00001
- Immunostained with anti-eNCAM


eNCAM
Control
20X


eNCAM
Acord-00001
20X

In Vitro Experiments

- E-18 cultures three weeks post treatment
- ***Vehicle treatment only***
- Immunostained with anti-β III Tubulin


β-tubulin
Control
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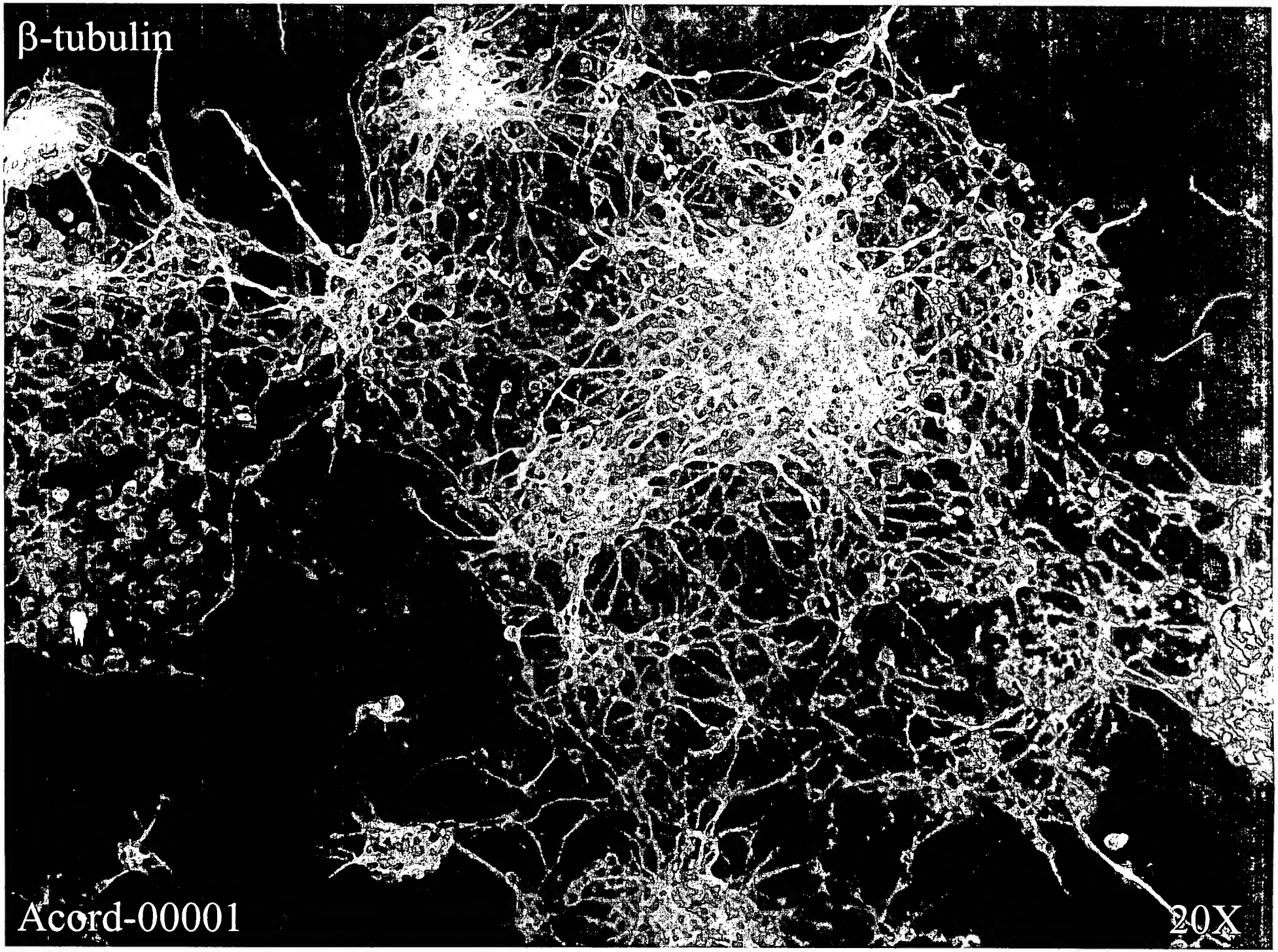
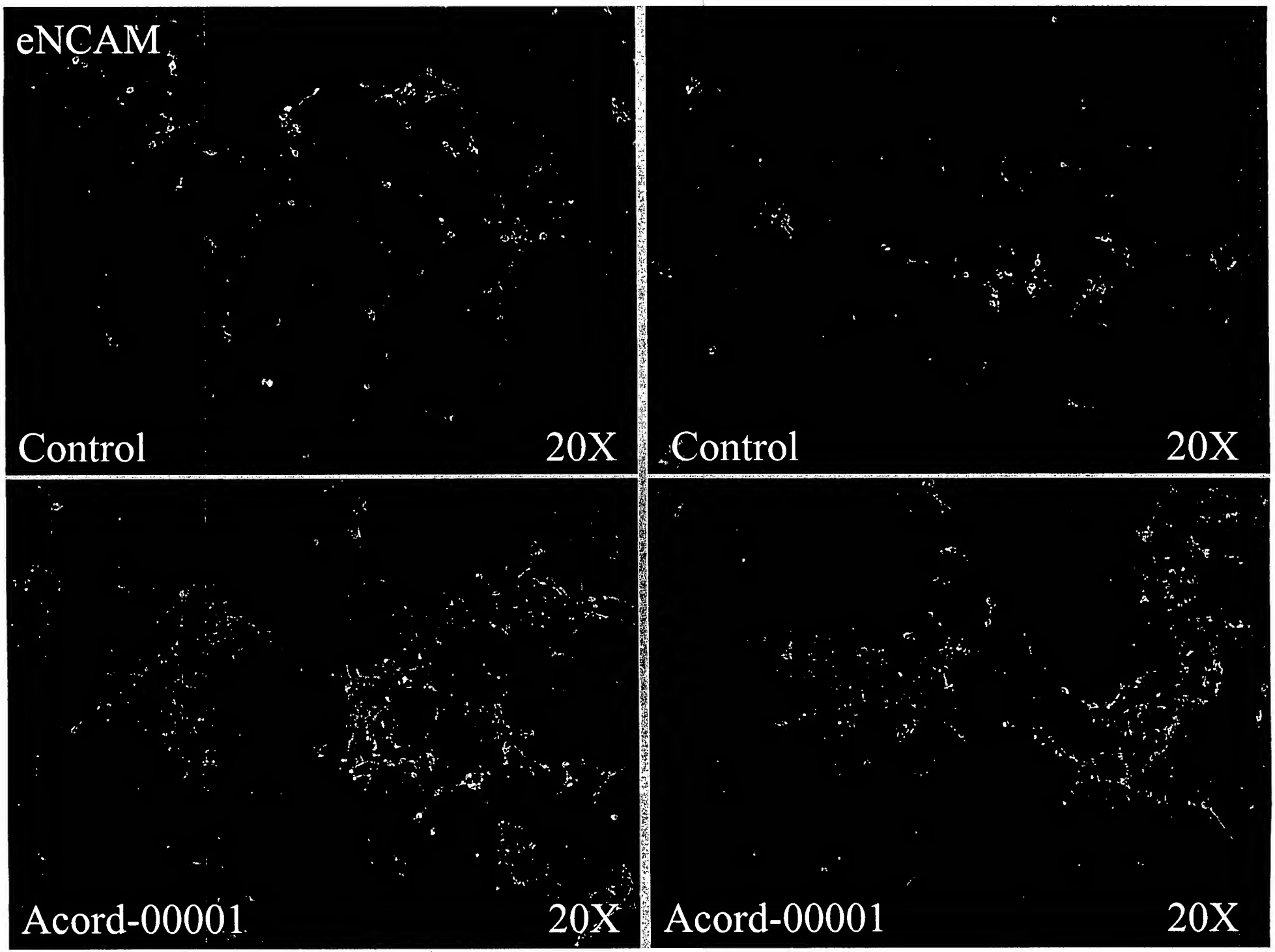
In Vitro Experiments

- E-18 cultures three weeks post treatment
- ***1 μg/ml Acord-00001***
- Immunostained with anti-β III Tubulin


β-tubulin
Acord-00001
20X

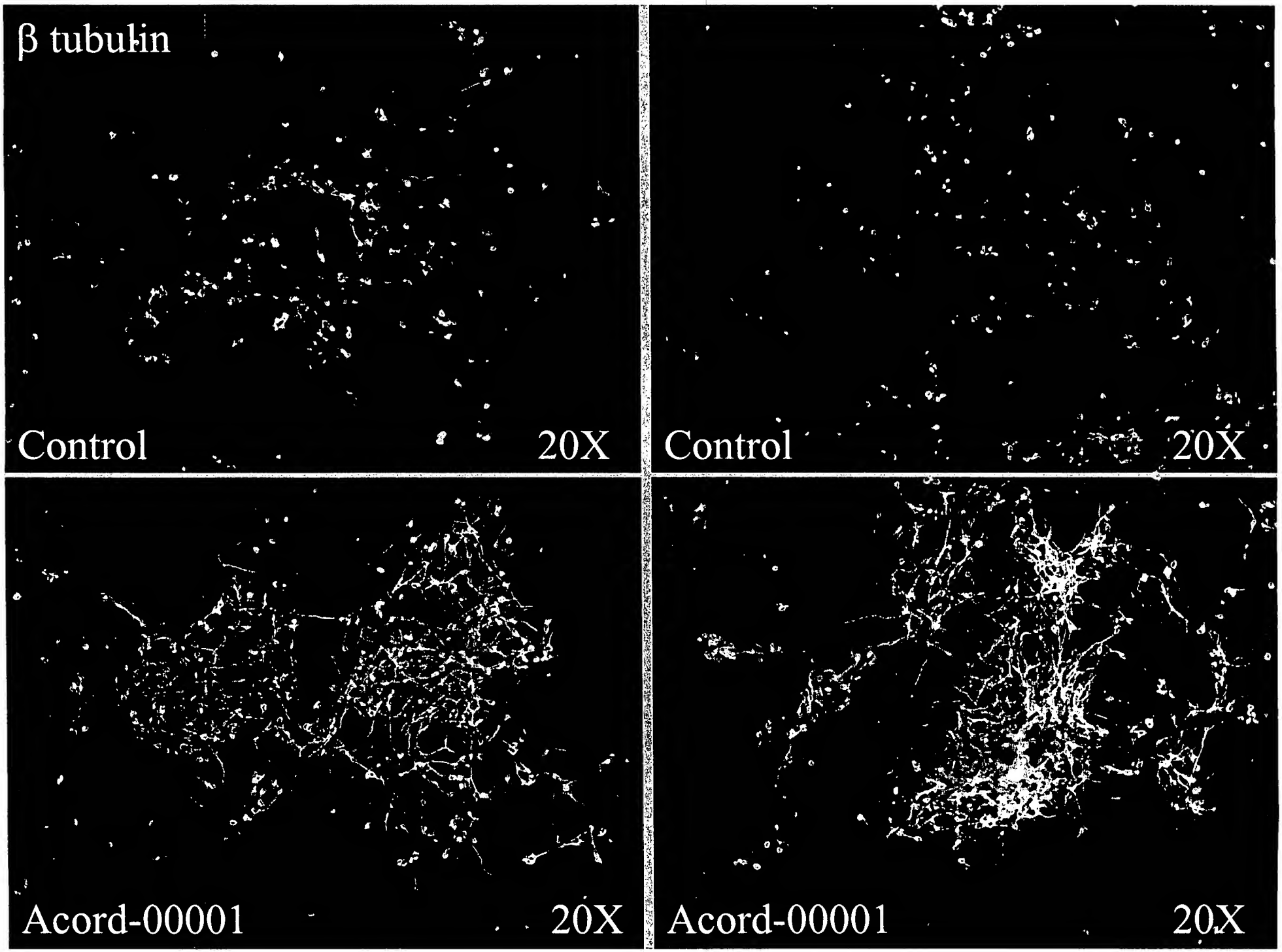
In Vitro Experiments

- Post natal day five, cultures one week treatment
- ***1 µg/ml Acord-00001***
- Immunostained with anti-eNCAM


eNCAM
Control
20X
Control
20X
Acord-00001
20X
Acord-00001
20X

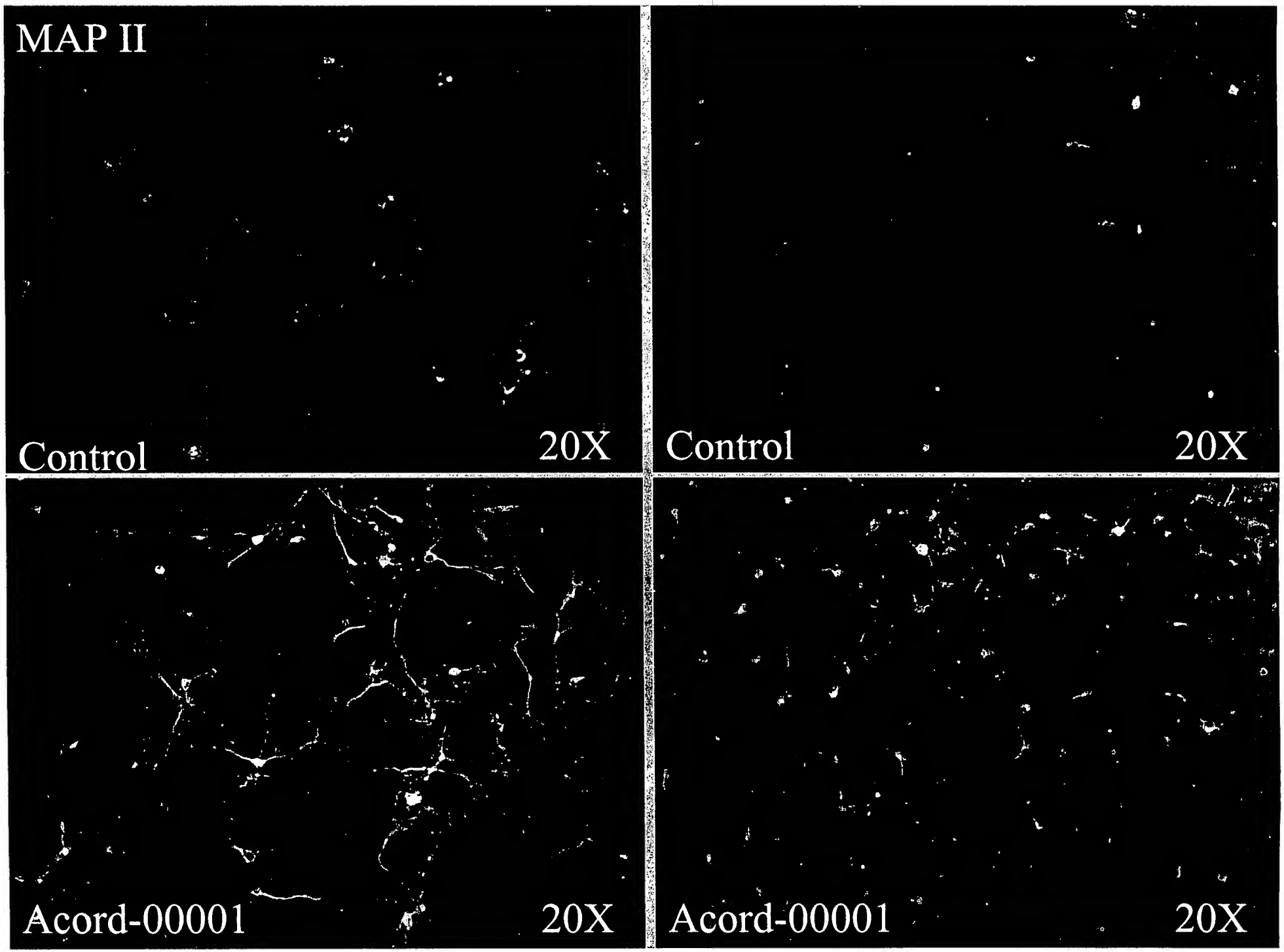
In Vitro Experiments

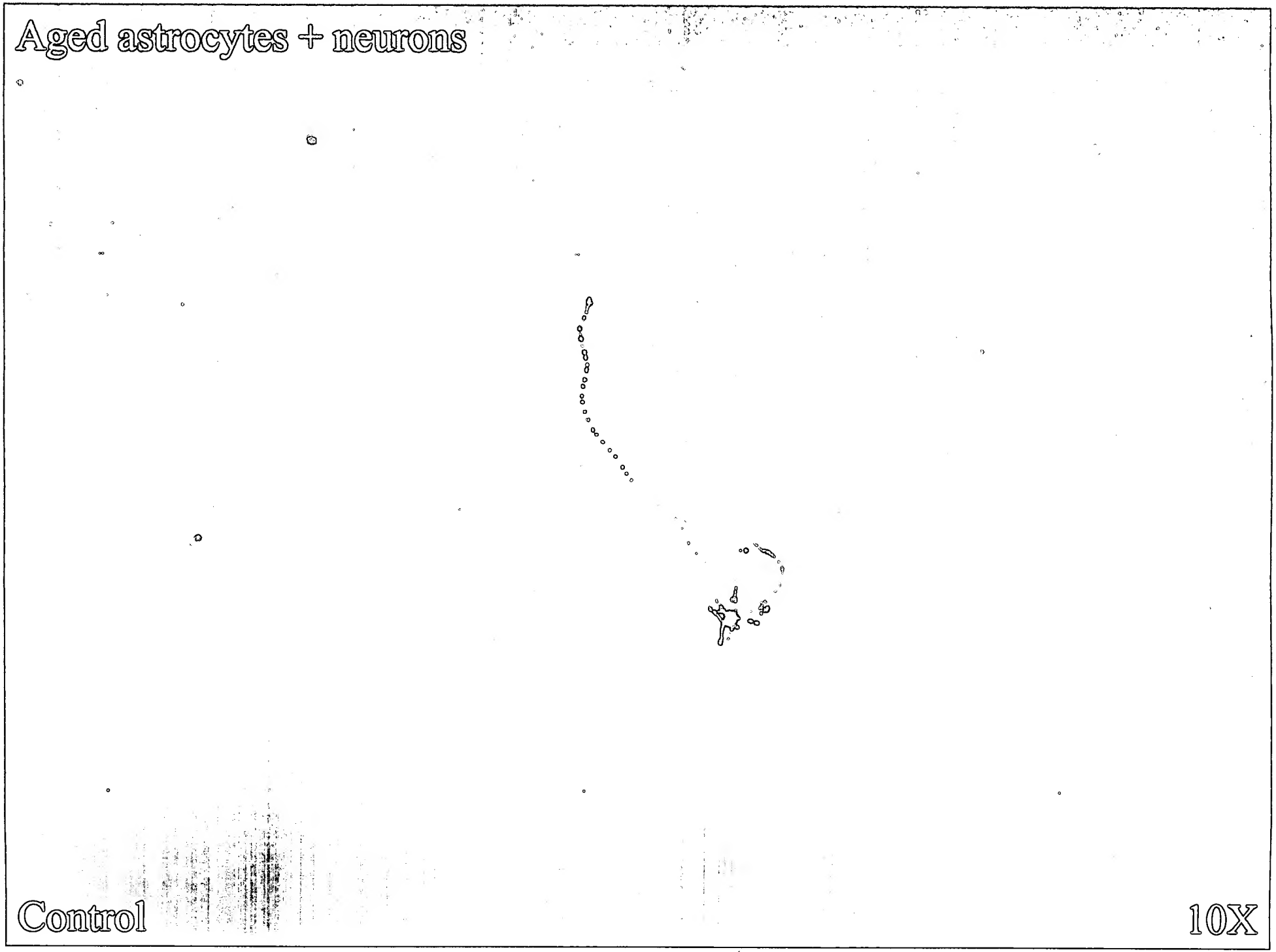
- Post natal day five, cultures four weeks post treatment
- ***1 μg/ml Acord-00001***
- Immunostained with anti-β tubulin

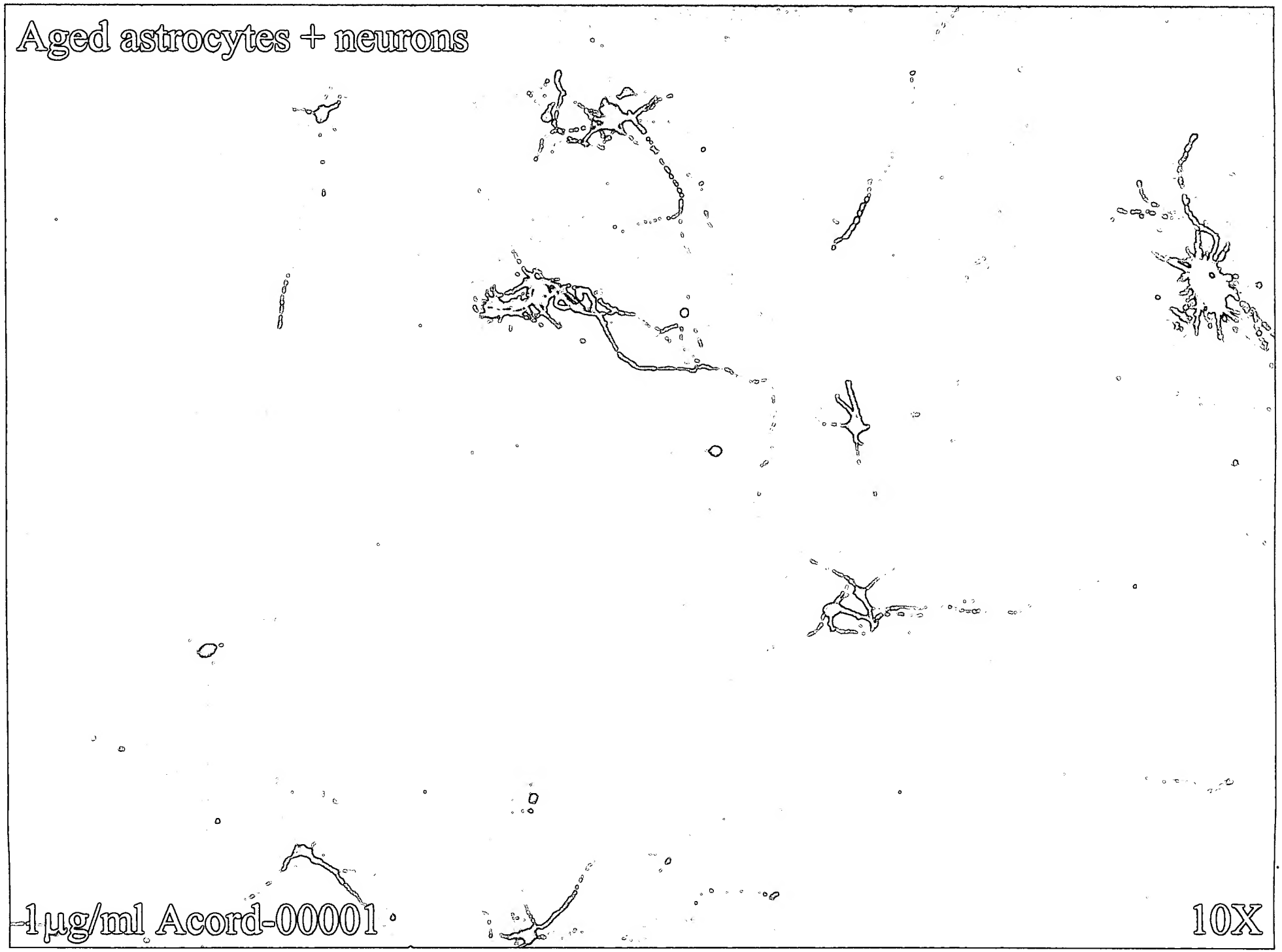

β tubulin
Control
20X
Control
20X
Acord-00001
20X
Acord-00001
20X

In Vitro Experiments

- Post natal day five, cultures twelve weeks post treatment
- ***1 µg/ml Acord-00001***
- Immunostained with anti-MAP II


MAP II
Control
20X
Control
20X
Acord-00001
20X
Acord-00001
20X


Aged astrocytes + neurons
Control
10X


Aged astrocytes + neurons
1µg/ml Acord-00001
10X

Non treated BM cells
Nestin-IR


Treated BM cells
Nestin-IR